Published in final edited form as:

J Comp Neurol. 2010 June 15; 518(12): 2365–2381. doi:10.1002/cne.22338.

Visual Deprivation Increases Accumulation of Dense Core Vesicles in Developing Optic Tectal Synapses in *Xenopus laevis*

Jianli Li and Hollis T. Cline*

Department of Cell Biology, The Scripps Research Institute, La Jolla, California 92037

Abstract

Despite considerable progress in understanding the molecular components of synapses in the central nervous system, the ultrastructural rearrangements underlying synaptic development remain unclear. We used serial section transmission electron microscopy and three-dimensional reconstructions of the optic tectal neuropil of *Xenopus laevis* tadpoles to detect and quantify changes in synaptic ultrastructure over a 1-week period from stages 39 and 47, during which time the visual system of *Xenopus* tadpoles becomes functional. Synapse density, presynaptic maturation index, and number of synapses per axon bouton increase, whereas the number of DCVs per bouton decreases, between stages 39 and 47. The width of the synaptic cleft decreased and the diameter of postsynaptic profiles increased between stages 39 and 47 and then remained relatively unchanged after stage 47. We found no significant difference in synapse maturation between GABAergic and non-GABAergic synapses. To test the effect of visual experience on synaptogenesis, animals were deprived of visual experience for 3 days from stage 42 to 47. Visual deprivation decreased synapse maturation and the number of connections per bouton. Furthermore, visual deprivation increased the number of DCVs per bouton by more than twofold. The visual-deprivation-induced decrease in synaptic connections is specific to asymmetric non-GABAergic synapses; however, both symmetric GABAergic and asymmetric synapses show comparable increases in the number DCVs with visual deprivation. In both the control and the visually deprived animals, the number of DCVs per bouton is highly variable and does not correlate with either synapse maturation or the number of connected partners per bouton. These data suggest that synaptogenesis and DCV accumulation are regulated by visual experience and further suggest a complex spatial and temporal relation between DCV accumulation and synapse formation.

Keywords

dense core vesicles; multiple synapse boutons; optic tectum; ultrastructure; 3D reconstruction; visual deprivation; synaptogenesis

---

Synaptogenesis requires the coordinated assembly of the pre- and postsynaptic elements, each of which is composed of a dense complex of proteins, lipids, and organelles. Despite considerable effort to describe the protein components of pre- and postsynaptic specializations and the time course of their assembly (Jin and Garner, 2008; McAllister, 2007), the cellular mechanisms underlying synaptogenesis are still not clear. Ultrastructural studies have demonstrated that key events in synaptic differentiation are the accumulation of synaptic vesicles in presynaptic terminals (Blue and Parnavelas, 1983; Dyson and Jones,

1980), the enlargement of the presynaptic active zone (Blue and Parnavelas, 1983; Dyson and Jones, 1980), and the accumulation of polyribosome (Steward and Falk, 1986). Other parameters such as the length of the postsynaptic density, the width of the synaptic cleft, and the diameter of the presynaptic terminals remain relatively unchanged (Vaughn, 1989). In addition, dense core vesicles (DCVs) are seen in ultrastructural samples at early stages of synaptogenesis (May and Biscoe, 1973; Vaughn, 1989) but are rarely seen in adult tissue (Sorra et al., 2006), leading to the suggestion by Vaughn (1989) that perisynaptic fusion of DCVs may be an essential feature of synaptogenesis.

DCVs are heterogeneous in their size and composition. Some DCVs include neuropeptides, growth factors, and synaptic vesicle proteins (Berg et al., 2000), and their fusion with the presynaptic membrane may release growth factors related to synaptogenesis and synaptic plasticity and may deliver components of the presynaptic active zone. Recent studies have shown by biochemical analysis that some DCVs include multiple molecular components of the presynaptic active zone, including the signature proteins, piccolo and bassoon. These vesicles are thought to transport the active zone components from the cell body to developing synapses, where their perisynaptic fusion may deliver a bolus of active zone components and allow rapid assembly of the presynaptic multi-protein complex (Bresler et al., 2004; Dresbach et al., 2006; Shapira et al., 2003; Zhai et al., 2001). Quantitative light microscopic analysis of synaptogenesis in cultured hippocampal neurons suggested that fusion of one or two bassoon-green fluorescent protein (GFP)-tagged transport vesicles is sufficient to produce a functional synapse (Shapira et al., 2003). Other studies combining in vivo light microscopic analysis of puncta visualized by expression of VAMP-GFP with retrospective electron microscopy suggested that a heterogeneous variety of membranous organelles including, but not limited to DCVs, could transport presynaptic proteins to developing synapses and participate in assembly of the presynaptic element (Ahmari et al., 2000). A recent study demonstrated by immunoelectron microscopy of developing ribbon synapses in the retina that bassoon is localized to nonmembranous, electron-dense "spheres" and suggests the presynaptic active zone proteins are transported as protein aggregates to the presynaptic terminal (Regus-Leidig et al., 2009). Therefore, the fundamental cellular events that generate functional synapses in the intact nervous system are still in question.

Here we conducted a quantitative ultrastructural analysis of synaptogenesis in the optic tectum of *Xenopus laevis* tadpoles, a system that has been well studied with respect to electrophysiological and light microscopic examination of synaptogenesis and synaptic plasticity. We quantified the developmental changes in synaptic organization during development of the retinotectal circuit, with particular attention paid to the presence and distribution of DCVs. Because their sparse distribution can lead to an underestimation of DCVs in single EM images, we generated 3D serial EM reconstructions through the synaptic neuropil and found that DCVs are up to eight times more abundant in synaptic boutons from younger animals than from older tadpoles. We tested, based on findings that synapse development is regulated by visual input (Aizenman and Cline, 2007; Sin et al., 2002), whether visual deprivation affects synaptogenesis of optic tectal synapses. Synapse maturation and the number of synaptic connections per bouton were decreased in the optic tectal neuropil of visually deprived animals, consistent with the idea that visual experience promotes synapse maturation. Although visual deprivation significantly increased the number of DCVs per axon bouton, DCVs do not accumulate selectively in immature synapses in either control tissue or tissue from visually deprived animals. These data suggest that visual experience affects synapse maturation and the distribution of DCVs.

MATERIALS AND METHODS

All experimental protocols were approved by the Cold Spring Harbor Laboratory Animal Care and Use Committee and complied with the guidelines established in the U.S. Public Health Service *Guide for the Care and Use of Laboratory Animals*.

Electron microscopy

Tadpoles of stages ranging from 39 to 56 (Nieuwkoop and Faber 1956) and a sexually mature male frog were used in this study. All tadpoles were from the same batch of fertilizations, and at least 10 animals were used from each stage. Animals were deeply anesthetized with 0.02% 3-aminobenzoic acid ethyl ester (MS222; Sigma, St. Louis, MO) in Steinberg's rearing solution, then fixed in a mixture of 2% paraformaldehyde, 2% glutaraldehyde, and 0.02% CaCl_2 in 0.035 M sodium cacodylate buffer at pH 7.4. To obtain optimal fixation, we pressure injected the fixative with 0.01% fast green into the brain ventricle with a Picospritzer until the entire tectum was surrounded by fixative. The frog was anesthetized with 0.5% MS-222 and perfused intravascularly with the same fixative. Both tadpole and frog brains were exposed to fixative and then immersed in the same fixative for 1 week at 4°C. The tadpole brains were rinsed in 0.1 M phosphate-buffered saline (PBS), pH 7.4; postfixed in 2% osmium tetroxide for 1 hour; dehydrated in an acetone series (50%, 70% with 1% uranyl acetate, 90%, 100%); and infiltrated with Epon 812 resin (50% in acetone, then 100% in acetone; Electron Microscopy Sciences, Fort Washington, PA). The frog brain was sectioned at 100 μm with a vibratome (Vibratome 3000), postfixed in 2% osmium tetroxide for 1 hour, and dehydrated and embedded as described above. On the next day, the tadpole brains and frog brain sections were flat embedded in 100% Epon 812 resin between two sheets of Aclar plastic (Ladd Research, Williston, VT) and incubated at 65°C overnight.

Serial sectioning electron microscopy and 3D reconstruction

We stained semithin horizontal sections (1 μm) through the tadpole brains with toluidine blue to identify regions of the optic tectum with similar dorsal ventral locations in the multiple brain samples. Ultrathin 70-nm serial sections (silver-gray interference color) were cut with a diamond knife (Diatome, Biel, Switzerland). All ultrathin sections were collected on formvar-coated nickel slot grids and stored in numbered boxes (Gilder grid storage box; Ted Pella, Redding, CA). Each grid can hold 10–50 sections. The ultrathin sections were examined with a Joel electron microscope 1010. Photographs were taken with an SIA CCD digital camera and sampled at $2,048 \times 2,048$ resolution. High-magnification photographs (10k or 30k) were taken for regions that contained synaptic contacts. We collected all our synapses in the neuropil layer of the optic tectum of tadpoles and frogs. We used the software developed by John Fiala (<http://synapses.bu.edu>) for 3D reconstruction. We reconstructed 59 axon boutons from three stacks of serial sections from stage 39 tadpoles and four stacks of serial sections from stage 47 tadpoles. We used Adobe Photoshop (Adobe systems, San Jose, CA) to produce all the figures. To make the lightest part of the background in electron photograph close to white, we adjusted the brightness of all electron micrographs in Adobe Photoshop.

Morphometric measurements and quantification of DCVs

To measure the width of the synaptic cleft, the distance across the cleft was measured at five locations within the synapse in a single EM section and averaged to give a single value for that synapse. To assess whether the distribution of synaptic contacts within the dendritic arbor changed over development, we estimated the diameter of pre- and postsynaptic profiles using an ellipse-fitting method in Image J. To quantify the presynaptic maturation index of synapses, we measured the areas of clustered synaptic vesicles and presynaptic

terminals using reconstruction software developed by John Fiala (<http://synapses.bu.edu>). In 3D-reconstructed axon boutons with multiple connected partners, the presynaptic maturation index is the average of all synapses. We also measured the volume of clustered synaptic vesicles and axon boutons with the same software.

We counted the number of DCVs in a complete sequence of aligned serial 70-nm EM sections through synaptic boutons. To guard against the possibility that variation in diameters of the DCVs could affect the result, we counted DCVs based on the location of the dense core. If a section is cut through the center of one dense core, it will only be counted as one because it will appear at the same location in two aligned consecutive sections. The values likely represent the absolute number of DCVs in fully reconstructed axon boutons.

Dissector analysis to estimate synapse density

Synapse density was determined by the dissector analysis method (Yen et al., 1993). At least 10 pairs of 70-nm sections at 1- μ m intervals were analyzed and synapses that appeared only in one of the two consecutive sections were counted. The synapse density was estimated as the sum of all counted synapses divided by the sum of the volume analyzed.

Immunohistochemistry

To detect the presence of GABA-immunoreactive synapses, we used postembedding immunocytochemical labeling (Li et al., 2003) with a polyclonal antibody that is made in rabbit using GABA conjugated with BSA as the immunogen (Sigma A2052) at a concentration of 1:500 and a secondary goat-anti-rabbit IgG conjugated to 15-nm gold particles (Amersham, Arlington Heights, IL). The GABA antibody has been extensively used in amphibians with labeling results consistent with ours (Bachy and Retaux, 2006; Moreno and Gonzalez, 2007). Double labeling with the GABA antibody and antibody to the vesicular transporter VGAT shows complete overlap (He and Cline, unpublished data).

Animals and visual deprivation

Tadpoles were reared on the laboratory bench at 22°C. The stages of tadpoles were identified according to their time postfertilization and morphological features (Nieuwkoop and Faber, 1956). Tadpoles were deprived of visual experience for 3 days from stage 42 to stage 47. They were put in a 60-mm petri dish (five tadpole per dish) and kept in a dark chamber for 3 days. Control tadpoles were reared with an approximately 12-hour dark/12-hour light cycle. Tadpoles then were fixed and processed as described above. We reconstructed 167 axon boutons from two stacks of serial sections from visually deprived tadpoles.

Statistical analysis

Data sets are expressed as mean $\pm$ SEM, and all error bars are SEM. The alpha of confidence level was set at 0.05. The statistical tests are indicated in Results.

RESULTS

Development changes in synaptic organization in optic tectum

We studied changes in the synaptic organization in the optic tectal neuropil of *Xenopus laevis* at different developmental stages between stage 39 (about 2.5 days after fertilization), when retinal axons first innervate the optic tectum (Holt and Harris, 1983), and stage 47 (around 6 days after fertilization), when the visual system is functional and controls behavior (Alsina et al., 2001; Bestman and Cline, 2008; Dong et al., 2009; Tao and Poo,

2005; Tao et al., 2001). Between stages 39 and 47, the optic tectum is composed of two major layers, a densely packed cell body layer and a neuropil layer, which includes neuronal axonal and dendritic processes and glial processes. At stage 39 (Fig. 1a–c), synapses can be identified in the neuropil layer. All the synapses we observed are axon–dendritic contacts: we do not see axosomatic synapses within the neuropil, because very few tectal cells have migrated into the neuropil layer at this stage. We did not observe synaptic vesicles in the postsynaptic profiles, and presynaptic terminals are not contacted by other presynaptic profiles, which suggests that the dendrodendritic synapses are very unlikely at stage 39. These nascent synapses have relatively few synaptic vesicles and postsynaptic densities in the dendritic profile opposite clustered synaptic vesicles. Other organelles, including mitochondria, were seen in pre- and postsynaptic sites. We found DCVs either distributed in presynaptic terminals or docked near the active zone. In addition, amorphous vesicular or vesiculotubular structures were seen in presynaptic terminals (Fig. 1a–e). As animals develop from stage 39 to stage 47, synapse density and the number of connections from single presynaptic terminals (Fig. 1d,e) increase. Inside presynaptic terminals, the number of synaptic vesicles increases and the number of DCVs decreases between stages 39 and 47. Occasionally, DCVs are observed in postsynaptic profiles (Fig. 1d). Extrasynaptic space, likely a result of the presence of extracellular matrix molecules that are invisible by standard EM methods, decreases between stages 39 and 47. With the adult frog, we examined the superficial layers of the optic tectal neuropil layers A–G (Potter, 1969) and found that clustered synaptic vesicles occupied most of area of the presynaptic terminals (Fig. 1f,g).

The developmental window between stages 39 and 47 is a period of rapid expansion of tectal cell dendritic arbors, retinal and mechanosensory axon innervation (Deeg et al., 2009; Hiramoto and Cline, 2009), and visual system developmental plasticity (Cline and Haas, 2008; Cline, 1998; Dong et al., 2009). We quantified the change in density and organization of synapses from both retinal and nonretinal inputs onto tectal cell dendrites during this period. Synapse density increases over threefold from stages 39 to 47 (Fig. 2a). To assess whether the distribution of synaptic contacts within the dendritic arbor changed over development, we estimated the diameter of pre- and postsynaptic profiles using an ellipse-fitting method. Although the diameter of presynaptic terminals did not change between stage 39 and 47 tadpoles and adult frog (Fig. 2b), the diameter of postsynaptic profiles increased significantly from stages 39 to 47, then was approximately the same in the adult frog (Fig. 2c), suggesting that synapses were located on larger dendritic processes in stage 47 tadpoles and in adults, compared with younger tadpoles. In addition, the width of the synaptic cleft gradually decreased during development (Kruskal-Wallis test, $H = 11.4$, $\chi^2 = 7.8$, $P < 0.05$. $P < 0.05$ between stages 39 and 47 with post hoc Mann-Whitney test), although no significant difference was detected after stage 47 (Fig. 3).

The most dramatic change in the development of synaptic morphology was the increase in the area occupied by clustered synaptic vesicles within presynaptic terminals between stages 39 and 47 and in adult frogs. To quantify this parameter, we defined the presynaptic maturation index of the synapse as the ratio of area that is occupied by clustered synaptic vesicles compared with the area of presynaptic terminal, as shown in Figure 2g,h. Here, “clustered” means that the neighboring synaptic vesicles appear to contact each other. The presynaptic maturation index increases steadily from stage 39 tadpoles to adult frog (Fig. 2d; median values: stage 39, 17.8; stage 47, 33.2; and adult, 57.6; Kruskal-Wallis test, $H = 48.0$, $\chi^2 = 6.0$, $P < 0.05$; $P < 0.01$ between stage 39 and 47; $P < 0.001$ between stage 47 and adult with post hoc Kolmogorov-Smirnov test; $n = 40$, 106, and 78 synapses from stage 39 and 47 tadpoles and adult frog, respectively). We compared the presynaptic maturation index determined from 2D electron micrographs with the ratio of volume of clustered synaptic vesicles with the volume of the axon boutons based on 3D reconstructions of axon boutons from stage 47 tadpoles. We found that the values are well correlated between the two

measurements ($R^2 = 0.859$, $n = 31$ boutons from stage 47 tadpoles; Fig. 2e). It is interesting to note that there is considerable variation in synapse maturation at each of the stages examined from stage 39 tadpole to the adult frog, shown by the wide distribution of the cumulative curve in Figure 2d. To test whether the variation in the increase in synapse maturation from stage 47 and adult frog might be due to differences in rates of synaptic maturation in different populations of synapses, we compared the presynaptic maturation index of synapses with symmetric and asymmetric contacts, which are likely inhibitory GABAergic and non-GABAergic synapses, respectively (see Fig. 4). We found that the presynaptic maturation indices of symmetric and asymmetric synapses are not different in stage 47 tadpoles or adults and that each type of synapse shows the same increase in presynaptic maturation index as the entire population (Fig. 2f).

Synaptic maturation in GABAergic and non-GABAergic neurons

Excitatory sensory inputs from the retina and hindbrain innervate the tectal neuropil, where they form synapses with both excitatory and inhibitory GABAergic tectal neurons (Akerman and Cline, 2006; Shen et al., 2009; Tao and Poo, 2005). Intratectal connectivity regulates sensory information processing in the tectum and governs tectal output (Dong et al., 2009; Tao and Poo, 2005). In addition to distinguishing GABAergic and non-GABAergic synapses based on the presence of symmetric and asymmetric synaptic connections (Fig. 2f), we also identified GABAergic terminals by using postembedding immunocytochemical labeling with gold particles to provide an independent assessment of whether synapse maturation differs between GABAergic and non-GABAergic synapses (Fig. 4a–e). To quantify the nonspecific background labeling in our samples, we first measured the density of gold particles in synapses with unambiguous asymmetric (non-GABAergic) contacts (4.0 ± 1.2 per μm^2 , $n = 14$). The profiles with at least five times the background signal were considered as GABAergic profiles. The size range of presynaptic terminals and postsynaptic dendritic profiles overlaps between the two types of synapses (Fig. 4f,g; $P = 0.70$ and 0.41 , respectively, Kolmogorov-Smirnov test). GABAergic and non-GABAergic synapses show a comparable distribution of presynaptic maturation index values at stage 47 (Fig. 4h; $P = 0.28$, Kolmogorov-Smirnov test, $n = 59$ GABAergic and $n = 45$ non-GABAergic synapses), consistent with the data on symmetric and asymmetric synapses (Fig. 2f). These results indicate that GABAergic and non-GABAergic synapses show a similar degree of developmental maturation at stage 47. The results also show that GABAergic and non-GABAergic inputs target similar dendritic regions of tectal neurons.

3D reconstructions of synaptic boutons reveal developmental changes in synaptic organization

We examined the development of synaptic connections in the optic tectum from 3D reconstructions of serial EM sections, because synaptic characteristics such as the number of synaptic connections from individual axon boutons and the number of DCVs per bouton can be underestimated in analysis of single EM sections. Presynaptic sites are located in axon boutons, which are enlarged, beaded structures along axon shafts, identified based on the presence of two narrow necks at either end of each bouton. The average number of connected partners per axon bouton increases from 1.2 ± 0.1 to 1.9 ± 0.1 from stage 39 to 47 ($P < 0.01$, Mann-Whitney test; $n = 18$ and 41 axon boutons; Fig. 5a–i). This shift in connectivity was also reflected in an increase in the percentage of synaptic boutons with multiple postsynaptic partners from 22% to 63%.

We quantified the number of DCVs in each completely reconstructed axon bouton. DCVs are easily distinguished from synaptic vesicles based on size (average diameter of DCVs: 67.6 ± 1.2 nm, range from 51 to 112 nm, $n = 86$; average diameter of synaptic vesicles: 39.2 ± 0.4 nm, range from 25 to 77 nm, $n = 184$; Fig. 6) and whether they have a dense or clear

interior. From stages 39 to 47, the average number of DCVs per axon bouton decreased from 26.4 ± 4.2 to 3.1 ± 0.5 ($P < 0.01$, $n = 18$ and 41 , Mann-Whitney test; Fig. 5j), whereas the diameter of DCVs remains relatively unchanged (average diameter of DCVs at stage 39 animal: 68.3 ± 2.2 nm, $n = 12$). These data suggest that the number of DCVs in axon boutons is inversely correlated with the density of synapses in stages 39 and 47, such that more DCVs are seen in boutons from stage 39 tadpoles, in which synapse density is low, and fewer DCVs per bouton are seen in stage 47 tadpoles, in which synapse density is higher.

The number of DCVs varies widely among axon boutons (Fig. 5j); however, we did not detect significant differences in the average number of DCVs in axon boutons with single synapse boutons (SSB) and those with multiple synapse boutons (MSB; 3.6 ± 1.0 vs. 2.9 ± 0.6 , $n = 15$ and 26 for SSBs and MSBs, $P = 0.89$, Mann-Whitney test; Fig. 5k) in stage 47 tadpoles. The number of DCVs per bouton is unlikely to be correlated with presynaptic maturation index of the axon bouton ($R^2 = 0.01$; Fig. 5l).

Visual deprivation increased accumulation of presynaptic DCVs

To test whether visual experience regulates synaptic organization in the developing optic tectum, we compared material from tadpoles deprived of visual experience for 3 days from stages 42 to 47 with material from tadpoles reared with an approximately 12-hour dark/12-hour light cycle. Although synapse density in the neuropil was not affected (control vs. visual deprivation: 0.56 ± 0.08 vs. 0.53 ± 0.10 synapse per μm^{-3} ; Fig. 7d), the presynaptic maturation index of synapses was lower in visually deprived animals compared with controls (Fig. 7e; $n = 106$ and 92 , $P < 0.05$, Kolmogorov-Smirnov test). Visual deprivation significantly decreased the average number of connections per bouton (control vs. visual deprivation: 1.9 ± 0.1 vs. 1.4 ± 0.1 , $n = 41$ and 167 , $P < 0.001$, Mann-Whitney test; Fig. 7f), which is also reflected in the increased proportion of single synapse boutons in visually deprived tadpoles (69% or $116/167$) compared with controls (37% or $15/41$).

The most dramatic effect of visual deprivation was an increase in the average number of DCVs per axon bouton, from 3.1 ± 0.5 in controls to 10.2 ± 1.2 in visually deprived animals ($n = 41$ and 167 , $P < 0.001$, Mann-Whitney test; Fig. 7a–c,g). There is a wide distribution in the number of DCVs per axon bouton with visual deprivation, ranging from 0 to 92 DCVs per bouton (Fig. 7g). Further analysis did not reveal a statistically significant accumulation of DCVs in SSBs compared with MSBs in visually deprived tadpoles (12.2 ± 1.7 vs. 5.9 ± 0.8 , $n = 116$ and 51 , $P = 0.25$ Mann-Whitney test; Fig. 7h). Again, the number of DCVs per bouton is not correlated with presynaptic maturation index of the synapses ($R^2 = 0.08$; Fig. 7i). In addition to the accumulation of DCVs in axon boutons (Figs. 7b,c, 8a–d), we found an increased distribution of DCVs in the axon shaft in visually deprived tadpoles, in which DCVs were often distributed along microtubules (Fig. 8e–g).

To test whether the changes in synaptic organization induced by visual deprivation occur selectively in excitatory or inhibitory synapses, we compared synapses with symmetric and asymmetric contacts, which, according to our GABA immunocytochemistry as well as published reports (Rybicka and Udin, 1994), represent inhibitory GABAergic and non-GABAergic synapses, respectively. Although the average number of DCVs in symmetric and asymmetric synapses is significantly greater in visually deprived animals compared with controls (symmetric: 2.7 ± 0.7 vs. 8.3 ± 2.4 ; asymmetric: 3.7 ± 0.8 vs. 9.7 ± 1.5 , control: $n = 13$ and 19 ; visually deprived: $n = 50$ and 62 for boutons with symmetric and asymmetric contacts, respectively; $P < 0.05$, Mann-Whitney test; Fig. 9a), as seen for the entire population of synapses (Fig. 7g), DCV numbers were not significantly different between GABAergic and non-GABAergic synapses. By contrast, only asymmetric, presumably glutamatergic contacts showed a significant decrease of number of connections per axon

bouton (2.0 ± 0.2 vs. 1.2 ± 0.1 , $n = 19$ and 62 for control and visually deprived animals; $P < 0.05$, Mann-Whitney test; Fig. 9b), whereas this measure of GABAergic connectivity was not altered with visual deprivation. These data suggest that visual deprivation causes a selective change in synaptic connectivity of asymmetric (excitatory) synapses in the tectum, which includes retinal inputs. By contrast, the increased presynaptic DCVs in both excitatory and inhibitory synapses (with both symmetric and asymmetric contacts) suggest that visual deprivation has a more circuit-wide effect on aspects of synaptic development relating to DCVs.

DISCUSSION

Quantification of synaptic development

The configuration of synaptic connections is thought to change rapidly during early stages of circuit development. Here we have examined synaptic development over a time window of 4 days of optic tectal development in *Xenopus* tadpoles, a time over which both retinal and mechanosensory axons first innervate the optic tectum and establish a circuit that processes sensory information and governs behavioral output (Alsina et al., 2001; Bestman and Cline, 2008; Deeg et al., 2009; Dong et al., 2009; Hiramoto and Cline, 2009; Tao and Poo, 2005; Tao et al., 2001). In agreement with reports of synaptic development in a variety of mammalian species (Blue and Parnavelas, 1983; Cragg, 1975; Dyson and Jones, 1980; Huttenlocher and Dabholkar, 1997; Rakic et al., 1986; Warton and McCart, 1989), we found a dramatic increase in synapse density as the tectal circuit develops; however, the increase in synapse density occurred over a period of a few days compared with weeks or months in the mammals that have been studied. The increased synapse density could be the result of two types of synapse formation: the addition of synaptic sites to preexisting boutons, to generate multisynapse boutons from single synapse boutons, and/or the formation of new axon boutons. We find that the percentage of multisynapse boutons in the optic tectum increases significantly from stage 39 to stage 47, demonstrating that this mechanism contributes to the increase in synapse density in the developing optic tectum, as has also been reported for the development of mammalian retinogeniculate and thalamo-cortical projections (Anderson et al., 1992; Lev et al., 2002). The formation of multisynapse boutons may provide higher interconnectivity with a relatively small volume and thereby permit greater structural plasticity (Stepanyants et al., 2002). Other studies have reported no change in percentage of multisynapse boutons in developing hippocampus (Fiala et al., 2002; Schikorski and Stevens, 1999). The different results could be due to different species, stages, or type of axon boutons.

Several recent studies have attempted to determine synaptic connectivity based on light microscopic detection of pre- and postsynaptic proteins using expression of fusion proteins or antibody labeling. The synaptic neuropil of the optic tectum is densely packed with fine-caliber postsynaptic profiles, which have a median diameter of about $0.2 \mu\text{m}$ at stage 39 and $0.5 \mu\text{m}$ at stage 47, and presynaptic boutons with a median diameter of less than $1 \mu\text{m}$. Furthermore, at stage 47, when the retinotectal circuit is very plastic, presynaptic boutons form an average of two synaptic connections. These features are also seen in developing CNS from zebrafish and mammalian species (Campbell and Shatz, 1992; Fiala et al., 1998; Jontes et al., 2000; Sorra and Harris, 1993; Sotelo, 2008; Yen et al., 1995). The ultrastructural data indicate that estimates of synaptic connectivity based on proximity of synaptic markers at the light microscopic level are likely to be unreliable and that the dream of visualizing synaptic dynamics in vivo is not possible with currently available light microscopic methods.

Presynaptic maturation index

Comparison of presynaptic terminals in adult frog and developing tadpoles indicated that a larger fraction of the presynaptic terminal area is occupied by clustered synaptic vesicles in adult tissue than in tadpoles. Therefore, we established a measure of synaptic maturation, called the “presynaptic maturation index,” based on the relative area occupied by clustered synaptic vesicles increases with development. We measured presynaptic maturation index as the ratio of area of clustered synaptic vesicles relative to the area of the presynaptic terminal and demonstrated that measurements made from single electron micrographs are well correlated with measurements made of the corresponding volumes based on 3D reconstructions of boutons. By using this indicator from single sections, we show that the presynaptic maturation index increased steadily during development, consistent with previous observations of a developmental increase in synaptic vesicles per terminal (Blue and Parnavelas, 1983; Dyson and Jones, 1980). At each stage that we studied, we found considerable variation in the presynaptic maturation index of synapses, which is reflected by the large variation in presynaptic terminal area occupied by synaptic vesicles among different synapses in our samples and other studies (Jones, 1983; Vaughn, 1989). Because the presynaptic maturation index is based on the relative area of clustered synaptic vesicles within the terminal, synapses with low maturation indices may be in the process of either synapse formation or synapse elimination. Nevertheless, synapses with high presynaptic maturation index values are likely to be strong connections with active synaptic transmission (Haas et al., 2006). Therefore, the variation in presynaptic maturation index suggests that, at each stage of circuit development, the population of synapses in the optic tectal neuropil is undergoing concurrent synaptogenesis, synapse maturation, and synapse elimination, as detected by in vivo time-lapse imaging (Alsina et al., 2001; Meyer and Smith, 2006; Ruthazer et al., 2006) and electrophysiological studies (Aizenman and Cline, 2007), and that individual synapses in the sample are caught at different points in this sequence during fixation.

Comparable developmental profiles of inhibitory and excitatory connections

The wide distribution of presynaptic maturation index values and numbers of connections per axon bouton could be due to heterogeneity of the synapses analyzed in our study. Several studies suggest that GABAergic contacts may develop before glutamatergic contacts in developing hippocampus (Hennou et al., 2002; Khazipov et al., 2001; Tyzio et al., 1999); however, other studies, including those of the *Xenopus* visual system, indicate that excitatory and inhibitory connections develop in concert (Akerman and Cline, 2006; Tao and Poo, 2005). Here we found that the populations of GABAergic and non-GABAergic synapses showed comparable distributions of synaptic maturation at stage 47, consistent with previous reports of simultaneous development of excitatory and inhibitory synapses in the *Xenopus* visual system (Akerman and Cline, 2006; Tao and Poo, 2005). Reports from rat visual cortex have shown comparable increases in synaptic vesicles between Gray type I and Gray type II synaptic contacts (Blue and Parnavelas, 1983), also indicating that GABAergic and non-GABAergic synapses develop in parallel. We used a postembedding technique with gold particles to identify GABAergic terminals in part because the variation in postsynaptic densities in young synapses makes it difficult to distinguish between symmetric and asymmetric synaptic contacts. Because we set the threshold of detection of GABAergic terminals at five times the background signal, we might have underestimated the number of GABAergic terminals with lower levels of GABA immunoreactivity; however, we believe that this is unlikely, because we detected many GABAergic synapses with low maturation indices. In conclusion, our data suggest that the increase in presynaptic maturation index is a general morphological change of both inhibitory and excitatory synapses in developing optic tectum.

Developmental changes in DCVs

DCVs are heterogeneous in their size and protein components, but they are generally distinguished as large DCVs (120 nm) or small DCVs (80 nm). Large DCVs contain neuroactive peptides, including neurotrophins (Huttner et al., 1995) and synaptic vesicle proteins (Berg et al., 2000). Some small DCVs are thought to contain molecular components of presynaptic active zones (Shapira et al., 2003; Zhai et al., 2001), but it is not clear whether small DCVs are a uniform population with respect to their composition and cargo. The distribution in size of both synaptic vesicles and DCVs in our EM samples is somewhat smaller than the values reported for synaptic vesicles or small DCVs from mammalian systems (Sorra et al., 2006; Zhai et al., 2001). This could be due to species differences or different tissue-processing methods. Nevertheless, the temporal correlation between the explosion of synapse formation and the rapid decrease in the numbers of DCVs between stages 39 and 47 support the idea that DCVs contribute to forming new synaptic connections (Sorra et al., 2006; Vaughn, 1989). Other studies, which tagged protein components of the presynaptic active zone (Cai et al., 2007; Dresbach et al., 2006; Fejtova et al., 2009; Tsurriel et al., 2009) or synaptic vesicles (Ahmari et al., 2000), suggested that presynaptic proteins are transported to nascent synaptic sites, but the ultrastructural identity of the organelle(s) that delivers presynaptic proteins is unclear and may include small DCVs (Ahmari et al., 2000; Sorra et al., 2006; Tao-Cheng, 2007); large DCVs, which have been shown to include synaptic proteins (Berg et al., 2000); and vesiculotubular structures (Ahmari et al., 2000), which are likely dynamic structures. It is also possible that presynaptic active zone proteins are delivered to the developing synapse by other organelles, such as the recently reported nonmembranous electron dense “transport sphere” (Regus-Leidig et al., 2009). At this point, further investigations are needed to determine the contents of DCVs in developing synapses and the organelles that contribute to the development of the synapse.

The number of DCVs per bouton is about eightfold higher at stage 39 compared with stage 47; however, several pieces of our data do not support the idea that location of DCVs clearly represents potential synaptic sites. First, the number of DCVs per bouton does not correlate with the presynaptic maturation index of the bouton. Second, the number of DCVs per bouton is comparable for MSBs or SSBs, even though SSBs are more likely sites of further synaptogenesis than MSBs. Finally, we rarely found DCVs outside of identifiable synaptic boutons, as might be expected if they accumulated at *de novo* sites of synaptogenesis or marked sites where a synapse would form (Sorra et al., 2006). Consistently with previous work (Jones, 1983; Vaughn, 1989), we found the majority of DCVs are distributed away from the active zone and are only occasionally attached to the presynaptic membrane within active zone (Figs. 2, 6). These results suggest that DCVs may be used to build up synaptic connections at preexisting or nascent axon boutons. As an alternate possibility, several studies have shown that presynaptic puncta are often sites of new axon branch initiation (Alsina et al., 2001; Javaherian and Cline, 2005; Meyer and Smith, 2006; Ruthazer et al., 2006). It would be intriguing to know whether axon boutons with a high density of DCVs serve as initiation sites for new axon branches.

Visual-deprivation-induced accumulation of DCVs

After axons of retinal ganglion cells innervate their postsynaptic targets, it is widely accepted that visual experience-dependent synaptic plasticity mechanisms sculpt the synaptic organization of retinotectal connections to generate a fully functional circuitry. A few days of visual deprivation delays the experience-dependent refinement of synaptic circuitry in mammals, assessed in electrophysiology or light microscopic studies (Hooks and Chen, 2006, 2008; Huberman et al., 2006; Katz and Shatz, 1996), mainly through the failure to eliminate weak synaptic connections; however, ultrastructural changes of synaptic connections during early visual deprivation remain largely unknown.

Here we quantified the deprivation-induced modifications in synaptic organization by using 3D serial EM reconstruction and found several novel changes in axon boutons with 3 days of visual deprivation: brief visual deprivation decreased synapse maturation, decreased the number of connected partners selectively for excitatory synaptic boutons, and increased the accumulation of DCVs per bouton. Because overall synapse density remains unchanged in visually deprived tadpoles, the increase in SSBs suggests that number of axon boutons likely increases. The decreased presynaptic maturation index of synapses could arise from a combined failure to eliminate weak synapses and to strengthen other synapses, insofar as both events are experience dependent (Chen and Regehr, 2000; Hooks and Chen, 2006, 2008). The increased number of DCVs in these boutons is also consistent with the idea that the initial stages of synapse formation are slowed in the absence of visual experience. It is interesting to consider that the decreased initiation of synapse formation may set up a condition for rapid synaptogenesis with a subsequent activity-dependent signal. Indeed, previous studies have shown that a 4-hour period of dark followed by a 4-hour period of visual stimulation induces formation of immature nascent synapses (Aizenman and Cline, 2007). The finding that SSBs and MSBs have similar numbers of DCVs suggests that visual deprivation results in comparable accumulation of DCVs independently of the number of synapses per bouton. This data suggest that subsequent visual experience could promote synaptogenesis at both types of boutons.

Acknowledgments

The authors thank Dr. Alev Erisir from the University of Virginia for her expert electron microscopy advice, Dr. Mitya Chklovski for help in aligning image stacks, the Cline Lab for helpful discussions, and Regina Faulkner for careful reading of the manuscript. We also thank the reviewers for constructive suggestions.

Grant sponsor: National Institutes of Health; Grant number: RO1 EY-011261 (to H.T.C.); Grant number: DP10D000458 (to H.T.C.).

LITERATURE CITED

- Ahmari SE, Buchanan J, Smith SJ. Assembly of presynaptic active zones from cytoplasmic transport packets. *Nat Neurosci.* 2000; 3:445–451. [PubMed: 10769383]
- Aizenman CD, Cline HT. Enhanced visual activity in vivo forms nascent synapses in the developing retinotectal projection. *J Neurophysiol.* 2007; 97:2949–2957. [PubMed: 17267761]
- Akerman CJ, Cline HT. Depolarizing GABAergic conductances regulate the balance of excitation to inhibition in the developing retinotectal circuit in vivo. *J Neurosci.* 2006; 26:5117–5130. [PubMed: 16687503]
- Alsina B, Vu T, Cohen-Cory S. Visualizing synapse formation in arborizing optic axons in vivo: dynamics and modulation by BDNF. *Nat Neurosci.* 2001; 4:1093–1101. [PubMed: 11593233]
- Anderson JC, Dehay C, Friedlander MJ, Martin KA, Nelson JC. Synaptic connections of physiologically identified geniculocortical axons in kitten cortical area 17. *Proc Biol Sci.* 1992; 250:187–194. [PubMed: 1283635]
- Bachy I, Retaux S. GABAergic specification in the basal forebrain is controlled by the LIM-hd factor Lhx7. *Dev Biol.* 2006; 291:218–226. [PubMed: 16438949]
- Berg EA, Johnson RJ, Leeman SE, Boyd N, Kimerer L, Fine RE. Isolation and characterization of substance P-containing dense core vesicles from rabbit optic nerve and termini. *J Neurosci Res.* 2000; 62:830–839. [PubMed: 11107168]
- Bestman JE, Cline HT. The RNA binding protein CPEB regulates dendrite morphogenesis and neuronal circuit assembly in vivo. *Proc Natl Acad Sci U S A.* 2008; 105:20494–20499. [PubMed: 19074264]
- Blue ME, Parnavelas JG. The formation and maturation of synapses in the visual cortex of the rat. II. Quantitative analysis. *J Neurocytol.* 1983; 12:697–712. [PubMed: 6619907]

- Bresler T, Shapira M, Boeckers T, Dresbach T, Futter M, Garner CC, Rosenblum K, Gundelfinger ED, Ziv NE. Postsynaptic density assembly is fundamentally different from presynaptic active zone assembly. *J Neurosci*. 2004; 24:1507–1520. [PubMed: 14960624]
- Cai Q, Pan PY, Sheng ZH. Syntabulin-kinesin-1 family member 5B-mediated axonal transport contributes to activity-dependent presynaptic assembly. *J Neurosci*. 2007; 27:7284–7296. [PubMed: 17611281]
- Campbell G, Shatz CJ. Synapses formed by identified retinogeniculate axons during the segregation of eye input. *J Neurosci*. 1992; 12:1847–1858. [PubMed: 1578274]
- Chen C, Regehr WG. Developmental remodeling of the retinogeniculate synapse. *Neuron*. 2000; 28:955–966. [PubMed: 11163279]
- Cline HT. Topographic maps: developing roles of synaptic plasticity. *Curr Biol*. 1998; 8:R836–R839. [PubMed: 9822571]
- Cline H, Haas K. The regulation of dendritic arbor development and plasticity by glutamatergic synaptic input: a review of the synaptotrophic hypothesis. *J Physiol*. 2008; 586:1509–1517. [PubMed: 18202093]
- Cragg BG. The development of synapses in the visual system of the cat. *J Comp Neurol*. 1975; 160:147–166. [PubMed: 1112924]
- Deeg KE, Sears IB, Aizenman CD. Development of multisensory convergence in the *Xenopus* optic tectum. *J Neurophysiol*. 2009 (in press).
- Dong W, Lee RH, Xu H, Yang S, Pratt KG, Cao V, Song YK, Nurmikko A, Aizenman CD. Visual avoidance in *Xenopus* tadpoles is correlated with the maturation of visual responses in the optic tectum. *J Neurophysiol*. 2009; 101:803–815. [PubMed: 19073807]
- Dresbach T, Torres V, Wittenmayer N, Altroch WD, Zamorano P, Zuschratter W, Nawroztzki R, Ziv NE, Garner CC, Gundel-finger ED. Assembly of active zone precursor vesicles: obligatory trafficking of presynaptic cytomatrix proteins Bassoon and Piccolo via a trans-Golgi compartment. *J Biol Chem*. 2006; 281:6038–6047. [PubMed: 16373352]
- Dyson SE, Jones DG. Quantitation of terminal parameters and their interrelationships in maturing central synapses: a perspective for experimental studies. *Brain Res*. 1980; 183:43–59. [PubMed: 7357409]
- Fejtova A, Davydova D, Bischof F, Lazarevic V, Altroch WD, Romorini S, Schone C, Zuschratter W, Kreutz MR, Garner CC, Ziv NE, Gundelfinger ED. Dynein light chain regulates axonal trafficking and synaptic levels of Bassoon. *J Cell Biol*. 2009; 185:341–355. [PubMed: 19380881]
- Fiala JC, Feinberg M, Popov V, Harris KM. Synaptogenesis via dendritic filopodia in developing hippocampal area CA1. *J Neurosci*. 1998; 18:8900–8911. [PubMed: 9786995]
- Fiala JC, Allwardt B, Harris KM. Dendritic spines do not split during hippocampal LTP or maturation. *Nat Neurosci*. 2002; 5:297–298. [PubMed: 11896399]
- Haas K, Li J, Cline HT. AMPA receptors regulate experience-dependent dendritic arbor growth in vivo. *Proc Natl Acad Sci U S A*. 2006; 103:12127–12131. [PubMed: 16882725]
- Hennou S, Khalilov I, Diabira D, Ben-Ari Y, Gozlan H. Early sequential formation of functional GABA(A) and glutamatergic synapses on CA1 interneurons of the rat foetal hippocampus. *Eur J Neurosci*. 2002; 16:197–208. [PubMed: 12169102]
- Hiramoto M, Cline HT. Convergence of multisensory inputs in *Xenopus* tadpole tectum. *Dev Neurobiol*. 2009 (in press).
- Holt CE, Harris WA. Order in the initial retinotectal map in *Xenopus*: a new technique for labelling growing nerve fibres. *Nature*. 1983; 301:150–152. [PubMed: 6823290]
- Hooks BM, Chen C. Distinct roles for spontaneous and visual activity in remodeling of the retinogeniculate synapse. *Neuron*. 2006; 52:281–291. [PubMed: 17046691]
- Hooks BM, Chen C. Vision triggers an experience-dependent sensitive period at the retinogeniculate synapse. *J Neurosci*. 2008; 28:4807–4817. [PubMed: 18448657]
- Huberman AD, Speer CM, Chapman B. Spontaneous retinal activity mediates development of ocular dominance columns and binocular receptive fields in v1. *Neuron*. 2006; 52:247–254. [PubMed: 17046688]
- Huttenlocher PR, Dabholkar AS. Regional differences in synaptogenesis in human cerebral cortex. *J Comp Neurol*. 1997; 387:167–178. [PubMed: 9336221]

- Huttner WB, Ohashi M, Kehlenbach RH, Barr FA, Bauerfeind R, Braunling O, Corbeil D, Hannah M, Pasolli HA, Schmidt A, et al. Biogenesis of neurosecretory vesicles. *Cold Spring Harb Symp Quant Biol.* 1995; 60:315–327. [PubMed: 8824405]
- Javaherian A, Cline HT. Coordinated motor neuron axon growth and neuromuscular synaptogenesis are promoted by CPG15 in vivo. *Neuron.* 2005; 45:505–512. [PubMed: 15721237]
- Jin Y, Garner CC. Molecular mechanisms of presynaptic differentiation. *Annu Rev Cell Dev Biol.* 2008; 24:237–262. [PubMed: 18588488]
- Jones DG. Recent perspectives on the organization of central synapses. *Anesth Analg.* 1983; 62:1100–1112. [PubMed: 6139964]
- Jontes JD, Buchanan J, Smith SJ. Growth cone and dendrite dynamics in zebrafish embryos: early events in synaptogenesis imaged in vivo. *Nat Neurosci.* 2000; 3:231–237. [PubMed: 10700254]
- Katz LC, Shatz CJ. Synaptic activity and the construction of cortical circuits. *Science.* 1996; 274:1133–1138. [PubMed: 8895456]
- Khazipov R, Esclapez M, Caillard O, Bernard C, Khalilov I, Tyzio R, Hirsch J, Dzhala V, Berger B, Ben-Ari Y. Early development of neuronal activity in the primate hippocampus in utero. *J Neurosci.* 2001; 21:9770–9781. [PubMed: 11739585]
- Lev DL, Weinfeld E, White EL. Synaptic patterns of thalamocortical afferents in mouse barrels at postnatal day 11. *J Comp Neurol.* 2002; 442:63–77. [PubMed: 11754367]
- Li J, Wang S, Bickford ME. Comparison of the ultrastructure of cortical and retinal terminals in the rat dorsal lateral geniculate and lateral posterior nuclei. *J Comp Neurol.* 2003; 460:394–409. [PubMed: 12692857]
- May MK, Biscoe TJ. Preliminary observations on synaptic development in the foetal rat spinal cord. *Brain Res.* 1973; 53:181–186. [PubMed: 4697244]
- McAllister AK. Dynamic aspects of CNS synapse formation. *Annu Rev Neurosci.* 2007; 30:425–450. [PubMed: 17417940]
- Meyer MP, Smith SJ. Evidence from in vivo imaging that synaptogenesis guides the growth and branching of axonal arbors by two distinct mechanisms. *J Neurosci.* 2006; 26:3604–3614. [PubMed: 16571769]
- Moreno N, Gonzalez A. Development of the vomeronasal amygdala in anuran amphibians: hodological, neurochemical, and gene expression characterization. *J Comp Neurol.* 2007; 503:815–831. [PubMed: 17570503]
- Nieuwkoop, PD.; Faber, J. Normal table of *Xenopus laevis* (Daudin). Elsevier-North Holland; Amsterdam: 1956.
- Potter HD. Structural characteristics of cell and fiber populations in the optic tectum of the frog (*Rana catesbeiana*). *J Comp Neurol.* 1969; 136:203–231. [PubMed: 5788725]
- Rakic P, Bourgeois JP, Eckenhoff MF, Zecevic N, Goldman-Rakic PS. Concurrent overproduction of synapses in diverse regions of the primate cerebral cortex. *Science.* 1986; 232:232–235. [PubMed: 3952506]
- Regus-Leidig H, Dieck S Tom, Specht D, Meyer L, Brandstatter JH. Early steps in the assembly of photoreceptor ribbon synapses in the mouse retina: the involvement of precursor spheres. *J Comp Neurol.* 2009; 512:814–824. [PubMed: 19067356]
- Ruthazer ES, Li J, Cline HT. Stabilization of axon branch dynamics by synaptic maturation. *J Neurosci.* 2006; 26:3594–3603. [PubMed: 16571768]
- Rybicka KK, Udin SB. Ultrastructure and GABA immunoreactivity in layers 8 and 9 of the optic tectum of *Xenopus laevis*. *Eur J Neurosci.* 1994; 6:1567–1582. [PubMed: 7850020]
- Schikorski T, Stevens CF. Quantitative fine-structural analysis of olfactory cortical synapses. *Proc Natl Acad Sci U S A.* 1999; 96:4107–4112. [PubMed: 10097171]
- Shapira M, Zhai RG, Dresbach T, Bresler T, Torres VI, Gundel-finger ED, Ziv NE, Garner CC. Unitary assembly of presynaptic active zones from Piccolo-Bassoon transport vesicles. *Neuron.* 2003; 38:237–252. [PubMed: 12718858]
- Shen W, Da Silva JS, He H, Cline HT. Type A GABA-receptor-dependent synaptic transmission sculpts dendritic arbor structure in *Xenopus* tadpoles in vivo. *J Neurosci.* 2009; 29:5032–5043. [PubMed: 19369572]

- Sin WC, Haas K, Ruthazer ES, Cline HT. Dendrite growth increased by visual activity requires NMDA receptor and Rho GTPases. *Nature*. 2002; 419:475–480. [PubMed: 12368855]
- Sorra KE, Harris KM. Occurrence and three-dimensional structure of multiple synapses between individual radiatum axons and their target pyramidal cells in hippocampal area CA1. *J Neurosci*. 1993; 13:3736–3748. [PubMed: 8366344]
- Sorra KE, Mishra A, Kirov SA, Harris KM. Dense core vesicles resemble active-zone transport vesicles and are diminished following synaptogenesis in mature hippocampal slices. *Neuroscience*. 2006; 141:2097–2106. [PubMed: 16797135]
- Sotelo C. Development of “Pinceaux” formations and dendritic translocation of climbing fibers during the acquisition of the balance between glutamatergic and gamma-aminobutyric acid inputs in developing Purkinje cells. *J Comp Neurol*. 2008; 506:240–262. [PubMed: 18022955]
- Stepanyants A, Hof PR, Chklovskii DB. Geometry and structural plasticity of synaptic connectivity. *Neuron*. 2002; 34:275–288. [PubMed: 11970869]
- Steward O, Falk PM. Protein-synthetic machinery at postsynaptic sites during synaptogenesis: a quantitative study of the association between polyribosomes and developing synapses. *J Neurosci*. 1986; 6:412–423. [PubMed: 3950704]
- Tao HW, Poo MM. Activity-dependent matching of excitatory and inhibitory inputs during refinement of visual receptive fields. *Neuron*. 2005; 45:829–836. [PubMed: 15797545]
- Tao HW, Zhang LI, Engert F, Poo M. Emergence of input specificity of Itp during development of retinotectal connections in vivo. *Neuron*. 2001; 31:569–580. [PubMed: 11545716]
- Tao-Cheng JH. Ultrastructural localization of active zone and synaptic vesicle proteins in a preassembled multi-vesicle transport aggregate. *Neuroscience*. 2007; 150:575–584. [PubMed: 17977664]
- Tsuriel S, Fisher A, Wittenmayer N, Dresbach T, Garner CC, Ziv NE. Exchange and redistribution dynamics of the cytoskeleton of the active zone molecule bassoon. *J Neurosci*. 2009; 29:351–358. [PubMed: 19144835]
- Tyzio R, Represa A, Jorquera I, Ben-Ari Y, Gozlan H, Aniksztejn L. The establishment of GABAergic and glutamatergic synapses on CA1 pyramidal neurons is sequential and correlates with the development of the apical dendrite. *J Neurosci*. 1999; 19:10372–10382. [PubMed: 10575034]
- Vaughn JE. Fine structure of synaptogenesis in the vertebrate central nervous system. *Synapse*. 1989; 3:255–285. [PubMed: 2655146]
- Warton SS, McCart R. Synaptogenesis in the stratum griseum superficiale of the rat superior colliculus. *Synapse*. 1989; 3:136–148. [PubMed: 2928962]
- Yen LH, Sibley JT, Constantine-Paton M. Fine-structural alterations and clustering of developing synapses after chronic treatments with low levels of NMDA. *J Neurosci*. 1993; 13:4949–4960. [PubMed: 8229207]
- Yen L, Sibley JT, Constantine-Paton M. Analysis of synaptic distribution within single retinal axonal arbors after chronic NMDA treatment. *J Neurosci*. 1995; 15:4712–4725. [PubMed: 7540683]
- Zhai RG, Vardinon-Friedman H, Cases-Langhoff C, Becker B, Gundelfinger ED, Ziv NE, Garner CC. Assembling the presynaptic active zone: a characterization of an active zone precursor vesicle. *Neuron*. 2001; 29:131–143. [PubMed: 11182086]

Figure 1. Ultrastructural morphology of synapses in the optic tectal neuropil during development. Electron micrographs are from tadpoles at stage 39 (**a–c**) and stage 47 (**d,e**) and from adult frog (**f,g**). Arrowheads mark synaptic sites, and stars mark dense core vesicles. Scale bar = 500 nm.

Figure 2.

Morphometric changes of synaptic features with development. **a**: Synapse density increased up to stage 47 and then decreased. **b**: The distribution of presynaptic terminal diameters for tectal synapses from stage 39 and 47 tadpoles and adult frog. **c**: The distributions of diameters of postsynaptic profiles increased significantly from stage 39 to 47 tadpoles, then remained similar in the adult frog. **d**: Cumulative distribution of presynaptic maturation index (PMI) of synapses in stage 39 and 47 tadpoles and adult frog. The median value (at 50%) and the distribution of presynaptic maturation index values increased steadily from stage 39 tadpole to adult frog. **e**: The presynaptic maturation index measured based on ratio of area and volume is well correlated. **f**: Developmental increase in presynaptic maturation index of synapses with clear symmetric and asymmetric contacts (stage 47: $n=44$ and 49 ; adult: $n=21$ and 29 synapses with symmetric and asymmetric contacts, respectively). **g,h**: Cartoon of a 3D reconstruction (**g**) and electron micrograph (**h**) showing the measurement of clustered synaptic vesicles (red), axon bouton (blue), and postsynaptic dendrite (green) based on volume and area, respectively. Scale bar = 500 nm.

Figure 3.

Developmental decrease in synaptic cleft width. **a–c:** Electron micrographs of synapses from stage 39 and 47 tadpoles and adult frog. **d:** The width of the synaptic cleft gradually decreased during development (stage 39, stage 41, stage 47, and adult: 21.0 ± 0.7 , 19.9 ± 0.5 , 18.3 ± 0.4 , 17.4 ± 0.5 nm; $n = 11, 9, 14, 17$). $*P < 0.05$. Scale bar = 100 nm.

Figure 4. GABAergic and non-GABAergic synapses show comparable developmental features. **a–e:** Electron micrographs of synaptic contacts from the optic tectal neuropil of stage 47 tadpoles. Presynaptic terminals with symmetric synaptic contacts were labeled with an antibody against GABA revealed by gold particles, as marked by white arrows. The terminals with asymmetric (non-GABAergic) synaptic contacts are marked by black arrows. **f–h:** Cumulative distribution curves demonstrate that medians and the range of diameters of postsynaptic profiles, presynaptic terminals, and presynaptic maturation index were not significantly different between GABAergic and non-GABAergic synapses. Scale bar = 1 μm .

Figure 5.

Morphometric analysis of axon boutons and DCVs from 3D reconstructions. **a,b**: 3D reconstructions of axon boutons from stage 39 and stage 47 tadpoles. The reconstructed axon boutons are represented in gray and white patches, representing the size and location of postsynaptic densities. **c–e**: Serial electron micrographs of synapses in the neuropil layer of optic tectum shown in **a**. **f–h**: Serial electron micrographs of synapses in the neuropil layer of optic tectum shown in **b**. Arrowheads mark synaptic sites, and stars mark dense core vesicles. **i**: The average number of connected partners per axonal bouton increased from stage 39 to 47. **j**: The average number of DCVs per bouton decreased from stage 39 to 47. Open circles represent number of DCVs in each bouton, and bars represent mean value. **k**: The average number of DCVs between SSBs and MSBs was not significantly different. **l**: Number of DCVs is not correlated with presynaptic maturation index of axon bouton. $*P < 0.05$. Scale bar = 500 nm.

Figure 6. Range of diameters of synaptic vesicles and dense core vesicles. Histogram of the distribution of diameters of synaptic vesicle and DCVs from synapses in the tectal neuropil of stage 47 tadpoles, binned at 5 nm.

Figure 7.

Visual deprivation increases presynaptic DCVs. **a–c:** 3D reconstruction of axon boutons from control (**a**) and visually deprived (**b,c**) tadpoles. Synaptic vesicles are represented by pink spheres; dense core vesicles are represented by green spheres; postsynaptic densities are represented by red patches. **d:** There was no significant difference in synapse density between visually deprived tadpoles and controls. **e:** The presynaptic maturation index of synapses was significantly decreased in visually deprived tadpoles. **f:** The average number of partners contacted by each axon bouton was decreased in visually deprived tadpoles. **g:** The average number of DCVs per axon bouton increased significantly in visually deprived tadpoles. **h:** The mean number of DCVs was not significantly different between SSBs and MSBs in visually deprived tadpoles. **i:** There was no apparent correlation between number of DCVs and presynaptic maturation index of axon boutons in visually deprived tadpoles. $*P < 0.05$. Scale box = $1 \mu\text{m}^3$.

Figure 8.

DCVs are found in the axon shaft of visually deprived tadpoles. **a**: 3D reconstruction of an axon bouton and adjoining axon shafts from a visually deprived tadpole. Dense core vesicles are represented by green spheres, and postsynaptic densities are represented by red patches. DCVs are present in the axon shaft. **b–d**: Electron micrographs of serial sections from visually deprived tadpoles showed the localization of DCVs at synaptic site marked in **a**. **e–g**: Serial sections showed that DCVs were distributed along microtubules in visually deprived tadpoles. Note the small caliber of the postsynaptic profile and the dense packing of the fine-caliber dendritic profiles. Scale box = 500 nm^3 in **a**; scale bar = 500 nm in **g** (applies to **b–g**).

Figure 9.

Selective decrease in synaptic connections from boutons with asymmetric contacts. **a:** The average number of DCVs per bouton increases in both GABAergic (symmetric) and non-GABAergic (asymmetric) boutons following 3 days of visual deprivation. **b:** Visual deprivation decreases the average number of connections in boutons with asymmetric contacts.